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Structural and functional analysis of *Trichoderma reesei* endoglucanase I expressed in yeast *Saccaromyces cerevisiae*

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The function of the domains of *Trichoderma reesei* endoglucanase I (EGI) has been studied. Truncated EGI proteins were expressed from the 3'-end deleted cDNAs in the yeast *Saccharomyces cerevisiae* under the control of the *ADC1* expression cassette. EGI protein was detected by monoclonal antibody EI-2 and EGI activity as cleared zones around growing colonies on agar plates containing hydroxyethylcellulose (HEC) covalently stained with Ostazin brilliant red (OBR). The results showed that the Thr–Ser-rich hinge region and the conserved 'tail' are not necessary for the efficient synthesis and secretion of EGI in yeast, but the intact core region is necessary for the enzymatic activity.

Cellulolytic yeast; Protein secretion; Endoglucanase functional domain; *Saccharomyces cerevisiae*; *Trichoderma reesei*

1. INTRODUCTION

Fungal and bacterial cellulases are composed of a core domain and a tail domain that contains a Thr–Ser- or Thr–Pro-rich B-region and a conserved A-region [1,2]. A distinct function has been suggested for each of these domains. The B-region is suggested to function as a flexible hinge between the two functional domains. The isolated core domain is able to degrade soluble cellulose [3]. Isolated tails have affinity for cellulose and are suggested to bind the enzyme to its substrate [4].

Genes for two cellobiohydrolases, CBHI and CBHII [5–7] and two endoglucanases, EGI and EGIII [8–10] have been isolated from *T. reesei*. The analysis of the gene sequence confirms the protein domain structure [11]. The domains of *T. reesei* CBHI and CBHII [12] and EGIII [3] and *Cellulomonas fimi* cellulases [13] can be separated proteolytically, but there are no reports concerning *T. reesei* EGI. Consequently, the function of the domains of EGI has not been studied directly although conclusions can be drawn from the sequence homology to other cellulases.

Because *T. reesei* produces a mixture of cellulases, the expression of cloned cDNA in *S. cerevisiae* provides a reliable method to study separately the properties of various cellulases or partial cellulase proteins. In this study series of 3'-end deleted EGI cDNAs were expressed in yeast and the secretion and the activity of the truncated EGI proteins were studied.

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2. MATERIALS AND METHODS

2.1. *Proteins and antibodies*

The purified EGI protein from *Trichoderma reesei* VTT-D-80133 [14] was a kind gift from Dr Marja-Liisa Niku-Paavola (Technical Research Centre of Finland, Espoo, Finland). Polyclonal antiserum KH1057 against EGI was prepared at the National Public Health Institute, Helsinki, Finland, by using their standard immunization procedure. The production and characterization of the monoclonal antibody EI-2 is described in [15].

2.2. *Construction of the cellulolytic yeast strains*

The standard recombinant DNA techniques described by Maniatis et al. [16] were used. The enzymes used in the cloning experiments were purchased from Boehringer Mannheim or New England Biolabs. *E. coli* DH5 [17] was used as a host for cloning the deleted cDNAs, which were prepared in vector pIBI76 (International Biotechnologies, Inc., New Haven, CT, USA). The deleted cDNAs were prepared as described earlier [18] and transferred to the yeast expression vector pAAH5 [19]. Plasmids containing the full length cDNAs for CBHI, CBHII, EGI and EGIII in pAAH5 (pALK220, pALK221, pALK222 and pALK223, respectively) and plasmids containing the selected, deleted cDNAs coding for truncated EGI were transformed [20] into *S. cerevisiae* strain Yf135 (*MATα*, leu2-3,112, his3-11,15). Yeast strains were grown in minimal medium containing 20 g glucose and 6.7 g yeast nitrogen base without amino acids (Difco) per liter and supplemented with amino acids except leucine in liquid culture, 1.5% agar was added for the solid support.

2.3. *Endoglucanase activity assay*

Recombinant yeast strains were tested for endoglucanase activity by growing the cells on the minimal agar plates as described above, supplemented with 0.5% (w/v) Ostazin brilliant red-hydroxyethyl cellulose (OBR-HEC, Sigma Chemical Co., St. Louis, MO, USA). The hydrolysis of OBR-HEC was detected as clear zones around the growing colonies on the red background [21].

2.4. *Filter immunodetection*

Yeast strains were streaked onto a nitrocellulose filter (Hybond C, Amersham, UK) covering the agar plate and grown at 30°C for 2–4 days. Filters were lifted from the plates and the cells washed off with

20 mM Tris-HCl, pH 7.5, 500 mM NaCl. The proteins on the filter were detected using the antibodies and the Protoblot Immunoblotting System (Promega, Madison, WI, USA) according to the manufacturer's instructions.

2.5. *Growth and fractionation of yeast cells*

The liquid cultures were grown at 30°C with shaking until the stationary phase was reached. Cells were separated by centrifugation for 15 min at 4000 × *g*. The proteins in the culture supernatant were precipitated with 10% trichloroacetic acid for 1 h on ice. The precipitate was collected by centrifugation, neutralized with ammonium vapour and dissolved in 250 mM sodium phosphate buffer pH 7.5, containing 50 mM EDTA, 1% (w/v) *n*-octylglucoside and 1% (v/v) 2-mercaptoethanol. Samples were incubated at 37°C overnight with one unit of *N*-glycosidase F (PNGase F, Boehringer, Mannheim Biochemica, Germany). The harvested cells were washed once, resuspended in 1/50 of the original volume of 50 mM Na-citrate buffer, pH 5.0, 1 mM phenylmethylsulphonyl-fluoride (PMSF, Sigma Chemical Co., St. Louis, MO, USA) and broken in a French pressure cell press (Aminco, Illinois, USA). The membrane fraction was separated from the soluble one by centrifugation at 10 000 × *g* for 15 min.

2.6. *Western blotting*

Proteins were separated on 10% polyacrylamide-SDS-gels [22] and transferred electrophoretically onto nitrocellulose membrane (BA 85, Schleicher and Schuell, Dassel, Germany) using the small scale electrophoresis and transfer system (Bio-Rad, Richmond, CA, USA). The membranes were immunostained with the monoclonal antibody EI-2, and developed using the Protoblot Immunoblotting System (Promega, Madison, WI, USA).

3. RESULTS

3.1. *The expression of cellulase cDNAs in yeast*

cDNAs for four *T. reesei* cellulases were expressed in *S. cerevisiae*. Their own signal sequences were used to conduct the secretion of the enzyme. The endoglucanase producing colonies formed a clear halo on the pink background. The yeast endogenous glucanases did not degrade the substrate used (Fig. 1A). Cellobiohydrolases secreted from yeast were not able to degrade HEC, but they were demonstrated by immunostaining with the cross-reacting polyclonal antiserum against EGI, KH1057 (Fig. 1B).

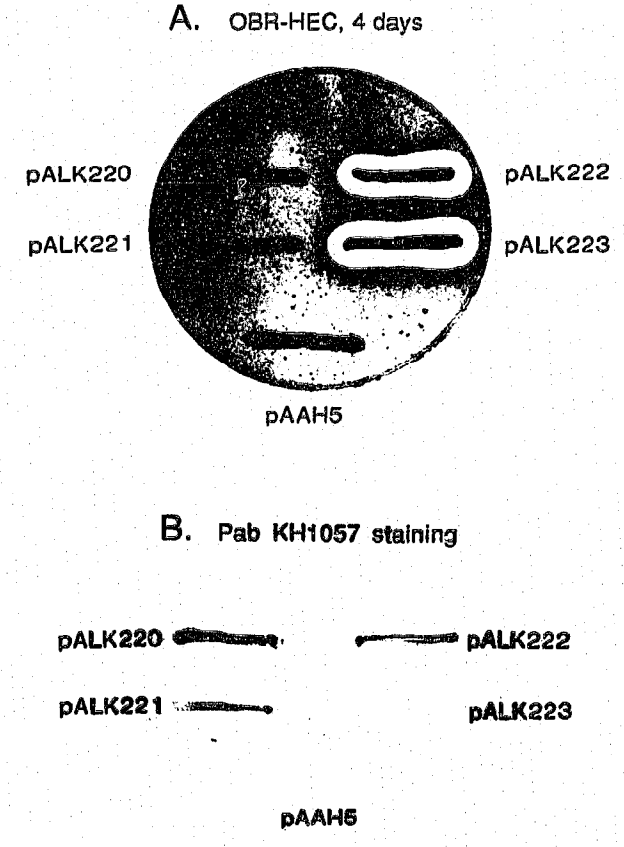

Fig. 1. Cellulolytic yeasts. Yeast strains expressing CBHI, CBHII, EGI and EGIII enzymes (see section 2) were grown for 4 days on a nutrient plate containing 0.5% OBR-HEC (A) or on the nitrocellulose filter covered plate (B). Hydrolysis of the substrate was documented by photographing the plate (A). The filter was immunologically stained using KH1057 antiserum (1/5000) (B) as described in section 2.

3.2. *The secretion of the truncated proteins*

The truncated proteins were expressed from the deleted cDNAs (Fig. 2). The deletion d2 lacked half of the 3′-end untranslated region but contained the intact EGI translation stop codon. The deletion d3 coded the full length protein, but lacked the translation stop codon and thus was expressed as a fusion protein containing the last 44 COOH-terminal amino acids of *ADC1*. Also d4 and d9 formed similar type of fusion proteins. Deletions d6, d7 and d10 were expressed as fusion proteins bearing 38 extra amino acids coded by the COOH-terminal region of the *ADC1* gene but not in the *ADC1* reading frame. Deletions d5 and d8 had only two extra amino acids before reaching the translation stop codon in the *ADC1* cassette. The proteins encoded by the full length cDNA and all the deletions except d10, were found in the particulate fraction of yeast cells (Fig. 3C). The soluble fraction contained proteins encoded by the full length cDNA and deletions d2, d3 and d4 (Fig. 3B). In addition to the protein encoded by the intact cDNA, the proteins encoded by deletions d2, d4 and d8 were found in the yeast culture medium (Fig. 3A). A weak band of proteins encoded by d3, d5, d7 and d9 could also be detected in the medium, but nothing was detected from d6 and d10.

3.3. *The enzymatic activity of the truncated EGI proteins*

The yeast strains containing the deleted EGI cDNAs were grown on the nutrient agar plate containing OBR-HEC as the substrate and indicator for the EGI enzyme activity (Fig. 4A,B). Deletion 4, which produced a polypeptide with the entire core region plus eight amino acids from the B-region, still made active enzyme, but deletion 5, producing a polypeptide missing 13 COOH-terminal amino acids from the core region did not produce active enzyme. The small hydrolysis halo around the d3 containing yeast strain was due to the inefficient

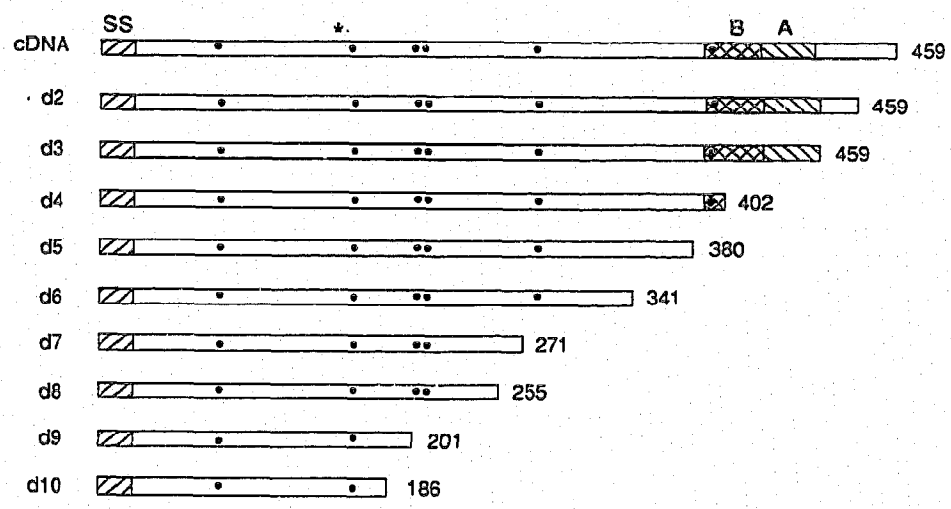

Fig. 2. Series of 3'-end deletions of EGI cDNA. The number of EGI amino acids in each protein encoded by the deleted cDNA is shown. The potential *N*-glycosylation sites are shown by heavy dots. The location of the active site is shown by an asterisk. The signal sequence (SS), the Thr–Ser-rich region (B), and the conserved region (A), are shown. The open box to the left of the BA domain represents the core domain and the open box to the right denotes the 3'-untranslated region of EGI cDNA.

secretion of the EGI enzyme (Fig. 3A). The type of the COOH-terminal fusion did not correlate with the efficiency of secretion of the truncated proteins. The secretion of the truncated proteins from the yeast cells was confirmed by immunostaining the nitrocellulose filter on which the yeast strains had grown for four days (Fig. 4C). The Mab EI-2 gave clear staining with all strains except d6 and d10. This is consistent with the Western blot (Fig. 3), which showed that d6 coded for an immunodetectable protein, which was not secreted. The protein encoded by d10 probably did not contain any antigenic epitope for Mab EI-2.

4. DISCUSSION

For the rapid screening of microorganisms producing and secreting endo-1,4-β-glucanase plate assays have been developed. The substrate polymer has been incorporated into the basal growth medium and the production of hydrolases is indicated by the clear zones of dissolved substrate around the colonies. Carboxymethylcellulose (CMC) and hydroxyethylcellulose (HEC), the substituted polymeric analogs of cellulose are generally used [23]. In the medium with CMC or β-glucan the detection of cellulases is accomplished by staining the residual substrate by Congo red [24]. When soluble HEC covalently dyed with OBR is mixed to agar nutrient media, the formation of pale clearing zones of the dissolved substrate around the growing colonies indicates the production of endoglucanases [21]. The yeast strains producing individual cellulases from the cloned cDNAs showed that endoglucanases possess considerable activity against hydroxyethylcellulose but cellobiohydrolases did not solubilize this substrate. The observation that CBHII contains some endoglucanase activity [25] was not confirmed in this study. The yeast endo-

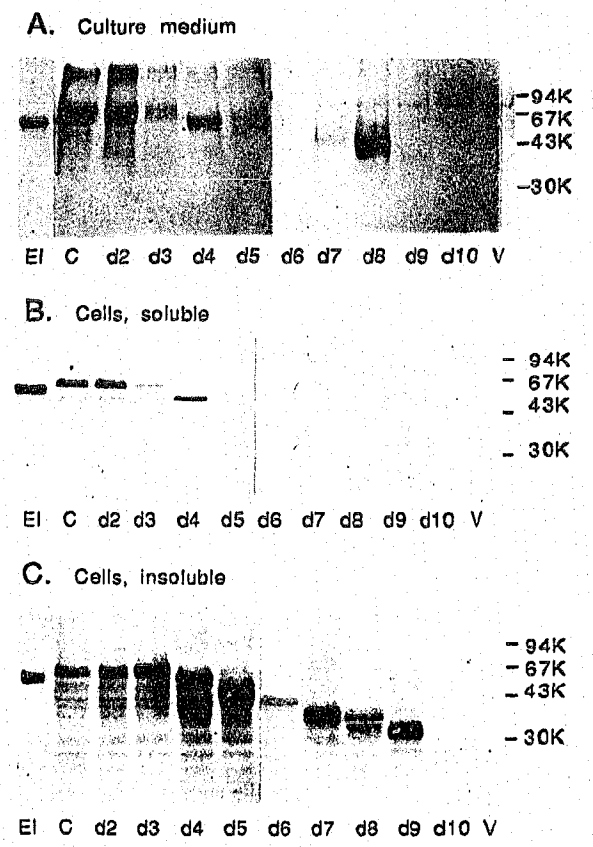

Fig. 3. Western blot analysis of proteins produced by yeast strains containing deleted EGI cDNA. Immunodetection was done with Mab EI-2, diluted 1:2500. EI, 30 ng of purified EGI; C, yeast strain with full length cDNA; V, yeast strain containing pAAH5; d2–d10, yeast strains containing deleted EGI cDNAs; A, culture medium concentrated from 4.5 ml and treated with PNGase F; B, cytoplasmic fraction from the cells, collected from 750 μl of stationary phase yeast culture; C, membrane fraction from the yeast cells, collected from 125 μl or from 300 μl (d6 and d8) of stationary phase yeast culture.

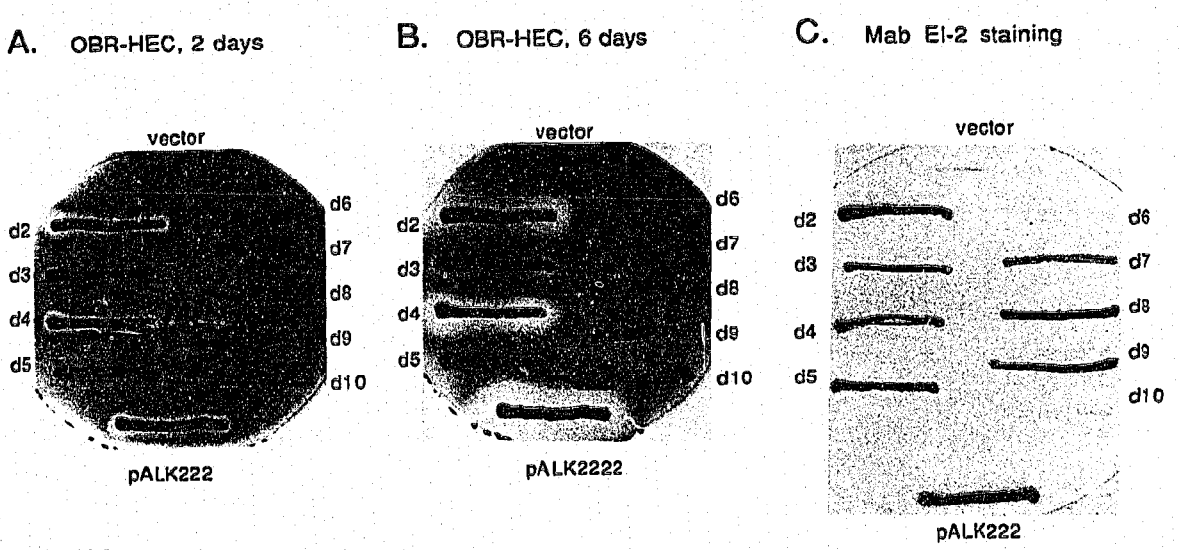

Fig. 4. EGI produced from the deleted cDNAs. Yeasts strains containing the full length EGI cDNA, pALK222, the expression vector pAAH5, and the deleted EGI cDNAs d2–d10 were grown on the plates containing 0.5% OBH-HEC at 30°C for 2 days (A) and for 6 days (B), at which time the size of the clearing was documented by photography. The proteins secreted from the yeast strains grown on the nitrocellulose filter covered plate were immunodetected using the Mab EI-2, diluted 1:2500.

genous glucanases did not show any activity against OBR-HEC. This method has been used in our laboratory for screening large number of yeast colonies for the increased EGI production after mutagenesis [26].

The Thr–Ser-rich domain of *Aspergillus awamori* glucoamylase I has been shown to function as a raw-starch-binding site [27]. In the case of yeast extracellular glucoamylase, encoded by *STA1*, mutant proteins without the Ser–Thr-rich region were not secreted but the hybrid β-galactosidase protein carrying the Ser–Thr-rich region was found transported to the cell envelope [28]. The analysis of the protein products from the intact and truncated genes coding for bacterial (*Bacillus, Clostridium*) endoglucanases has shown that the 3'-half of the gene can be removed and the remaining 5'-half still codes for an active enzyme, which is efficiently secreted [29–31]. In this work, the truncated EGI proteins lacking the Thr–Ser-rich B-region, encoded by d2 to d9, were found in the cell membrane fraction but only the EGI proteins containing the Thr–Ser-rich region or part of it were found in the soluble fraction of yeast cells. Because the whole cells were fractionated, the soluble fraction contained both the cytoplasmic and periplasmic soluble proteins. Proteins encoded by cDNA deletions d5, d7 and d8 were found secreted into the culture medium. This suggests that the secretion of *T. reesei* endoglucanase I from *S. cerevisiae* does not involve any specific linear amino acid sequence in addition to the amino terminal signal sequence, but most probably results from the favourable folding of protein. The secretion of the truncated proteins did not either correlate to the number of the putative *N*-glycosylation sites on each truncated protein.

Cellulomonas fimi cellulases show considerable conservation of functional domains with *T. reesei* cellulases [1]. The gene regions coding for the catalytic domains ('core' region) of the exoglucanase (Exg) and the endoglucanase (EngA) has been fused and expressed in *E. coli* [32]. The active bifunctional fusion protein was obtained; even part of the endoglucanase 'core' region became deleted as a result of the gene fusion method used. The intact core region of *T. reesei* EGI has been shown in this work to be necessary for the enzymatic activity. Because the putative active site is located about 200 amino acids away from the hinge region, the loss of activity after the removal of the 13 COOH-terminal amino acids of the core region can be due to the improper folding.

The 255 amino acids long EGI protein was detected in the yeast culture medium by using the monoclonal antibody EI-2. This suggests that part of the EGI can be used as a convenient marker for a variety of fusion proteins when studying their secretion pathway in yeast. On the other hand, the intact core region is essential to obtain the enzymatically active *T. reesei* EGI, expressed in *S. cerevisiae*.

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REFERENCES

[1] Knowles, J., Lehtovaara, P. and Teeri, T. (1987) Trends Biotechnol. 5, 255–261.
[2] Warren, R.A.J., Beck, C.F., Gilkes, N.R., Kilburn, D.G., Langsford, M.L., Miller, R.C., Jr., O'Neill, G.P., Scheufens, M. and Wong, W.K.R. (1986) Proteins 1, 335–341.
[3] Ståhlberg, J., Johansson, G. and Pettersson, G. (1988) Eur. J. Biochem. 173, 179–183.
[4] Ong, E., Greenwood, J.M., Gilkes, N.R., Kilburn, G., Miller, R.C., Jr. and Warren, R.A.J. (1989) Trends Biotechnol. 7, 239–243.

[5] Shoemaker, S., Schweickart, V., Lander, M., Gelfand, D., Kwok, S., Myambo, K. and Innis, M. (1983) Bio/Technology 1, 691–696.
[6] Teeri, T., Salovuori, I. and Knowles, J. (1983) Bio/Technology 1, 696–699.
[7] Teeri, T.T., Lehtovaara, P., Kauppinen, S., Salovuori, I. and Knowles, J. (1987) Gene 51, 43–52.
[8] Penttilä, M., Lehtovaara, P., Nevalainen, H., Bhikhabhai, R. and Knowles, J. (1986) Gene 45, 253–263.
[9] Van Arsdell, J.N., Kwok, S., Schweickart, V.L., Ladner, M.B., Gelfand, D.H. and Innis, M.A. (1987) Bio/Technology 5, 60–64.
[10] Saloheimo, M., Lehtovaara, P., Penttillä, M., Teeri, T.T., Ståhlberg, J., Johansson, G., Petterson, G., Claeyssens, M., Tomme, P. and Knowles, J. (1988) Gene 63, 11–21.
[11] Abuja, P.M., Pilz, I., Claeyssens, M. and Tomme, P. (1988) Biochem. Biophys. Res. Commun. 156, 180–185.
[12] Tomme, P., Van Tilbeurgh, H., Pettersson, G., Van Damme, J., Vandekerckhove, J., Knowles, J., Teeri, T. and Glaeyssens, M. (1988) Eur. J. Biochem. 170, 575–581.
[13] Gilkes, N.R., Warren, A.J., Miller, R.C., Jr. and Kilburn, D.G. (1988) J. Biol. Chem. 263, 10401–10407.
[14] Niku-Paavola, M.-L., Lappalainen, A., Enari, T.-M. and Nummi, M. (1985) Biochem. J. 231, 75–81.
[15] Aho, S., Olkkonen, V., Jalava, T., Paloheimo, M., Bühler, R., Niku-Paavola, M.-L., Bamford, D. and Korhola, M. (1991) Eur. J. Biochem., in press.
[16] Maniatis, T., Fritsch, E.F. and Sambrook, J. (1982) Molecular Cloning. Cold Spring Harbor Laboratory, New York.
[17] Hanahan, D. (1983) J. Mol. Biol. 166, 557–580.
[18] Aho, S. and Paloheimo, M. (1990) Biochim. Biophys. Acta 1087, 137–141.
[19] Ammerer, G. (1983) Methods in Enzymology (Wu, R., Grossman, L. and Moldave, K. eds) 101 C, pp. 192–201. Academic Press, New York.
[20] Ito, H., Fukuda, Y., Murata, K. and Kimura, A. (1983) J. Bacteriol. 153, 163–168.
[21] Farkas, V., Liskova, M. and Biely, P. (1985) FEMS Microbiol. Lett. 28, 137–140.
[22] Laemmli, U.K. (1970) Nature 227, 680–685.
[23] Penttillä, M.E., André, L., Saloheimo, M., Lehtovaara, P. and Knowles, J.K.C. (1987) Yeast 3, 175–185.
[24] Wood, P.J. (1980) Carbohydr. Res. 85, 271–287.
[25] Kyriacou, A., MacKenzie, C.R. and Neufeld, R.J. (1987) Enzyme Microbiol. Technol. 9, 25–32.
[26] Arffman, A., Aho, S., Torkkeli, H. and Korhola, M. (1990) Yeast 6 (Spec. Iss.), S438.
[27] Hayashida, S., Kuroda, K., Ohta, K., Kuhara, S., Fukuda, K. and Sakani, Y. (1989) Agric. Biol. Chem. 53, 923–929.
[28] Yamashita, I. (1989) Agric. Biol. Chem. 53, 483–489.
[29] Fukumori, F., Kudo, T. and Horikoshi, K. (1987) FEMS Microbiol. Lett. 40, 311–314.
[30] Lo, A.C., MacKay, R.M., Seligy, V.L. and Willick, G.E. (1988) Appl. Environm. Microbiol. 54, 2287–2292.
[31] Hall, J., Hazelwood, G.P., Barker, P.J. and Gilbert, H.J. (1988) Gene 69, 29–38.
[32] Warren, R.A.J., Gerhard, B., Gilkes, N.R., Owolabi, J.B., Kilburn, D.G. and Miller, R.C., Jr. (1987) Gene 61, 421–427.